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NOVEL COMPOUNDS

The present invention is related to an antibody, or fragments thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells; and to a target structure displayed in, or on the surface of tumour cells; a vaccine composition comprising said target structure; a pharmaceutical composition comprising said antibody; as well as methods related to human malignant diseases.

BACKGROUND OF THE INVENTION

Surgery is the primary treatment of colorectal cancer leading to five-year survival rates of 90 to 40 percent depending on the state of tumour progression from Dukes Stage A to C. Conventional adjuvant therapy that includes radiation therapy and chemotherapy has been able to reduce the death rates further by approximately 30 percent (1). Despite these achievements cancer of the colon and rectum is one of the major causes of death in human cancer. Immunological therapy has been extensively attempted. However, colon cancer has generally been resistant to immunotherapy and is considered to be of low immunogenicity. Patients with colon cancer neither respond to IL-2 treatment or adoptive transfer of *in vitro* cultured tumour infiltrating lymphocytes otherwise active in patients with immunogenic malignancies such as melanoma. Most encouraging however, Riethmüller et al. reported a 32 percent decreased seven-year death rate for Dukes Stage C colorectal cancer treated after primary tumour resection with a naked murine mAb directed to a tumour and normal epithelial associated antigen (Ep-CAM) (2), indicating that other immunotherapeutic modalities could be effective.

A significant improvement of adjuvant immunotherapy and of the treatment of more advanced stages of canc r should require a more potent effector mechanism than provided by a naked mAb. In principle, an increased potency should require an increased tumour selectivity of the targeting antibody.

The limited number of colon cancer associated antigens defined today have been discovered using hybridoma produced murine mAbs resulting from xenogenic immunisations with human tumours (3).

The use of large phage display libraries for the identification of novel tumour-associated antigens can be expected to significantly speed up the process of finding target molecules useful for tumour immunotherapy and diagnosis. Such identification of target molecules could be accomplished by the selection and screening of antibody phage libraries on cultured tumour cells and tissue sections to generate specific reagents defining *in vitro* and *in vivo* expressed antigens (4). The phage display technology has been established as an efficient tool to generate monoclonal antibody reagents to various purified antigens, and the construction and successful selection outcome from immune, naive and synthetic antibody phage libraries have been described in several studies (5).

Non-immune libraries are favourable with respect to their general applicability, making unique libraries for every single target unnecessary. On the other hand, sufficiently large and high quality non-immune libraries are difficult to construct and a target discovery process using these libraries should require efficient subtractive selection methods when based on complex antigens.

A phage library of a more moderate size has now been constructed from a near human primate immunised with complex human antigens. This represents an approach that takes advantage of an in vivo pre-selected repertoire. Such libraries should be enriched for specificities to

tumour specific epitopes in a reduced background reactivity to xenogeneic antigens (6). Furthermore, as compared to the mouse, primate antibodies demonstrating close sequence homology with human antibodies should not be immunogenic in man (7).

Novel primate antibodies from a phage library that define selectively expressed colon cancer associated antigens have now been identified. The therapeutic potential, demonstrated by T cell mediated killing of cultured colon cancer cells coated with two of these antibodies fused to engineered superantigens, is comparable with superantigens fused to murine Fab fragment specific for colon cancer associated antigens such as EP-CAM, for which there has previously been established the therapeutic capacity in experimental systems (8).

There is also provided a method for efficient positive and subtractive cell selection of phage antibodies that should facilitate future identification of novel phenotype specific antigens including tumour associated antigens using antibodies from large phage libraries.

BRIEF SUMMARY OF THE INVENTION

The present invention is related in a first aspect to an antibody, or fragments thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells, said binding structure comprising the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:1, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence

shown in SEQ ID NO: 1, or other binding structures with similar unique binding properties.

In one embodiment the antibody is phage selected. In another embodiment the sequences are of Macaca fascicularis origin, which sequences have an identity of at least 84% to corresponding sequences of human origin. Preferably, the antibody has low immunogenicity or non-immunogenicity in humans.

In a further embodiment, the antibody has been derivatised by genetically linking to other polypeptides, and/or by chemical conjugation to organic or non-organic chemical molecules, and/or by di-, oligo- or multimerisation.

In still a further embodiment, said antibody is genetically linked or chemically conjugated to cytotoxic polypeptides or to cytotoxic organic or non-organic chemical molecules.

In a further embodiment, said antibody is genetically linked or chemically conjugated to biologically active molecules.

In still a further embodiment, said antibody is genetically linked or chemically conjugated to immune activating molecules.

In another embodiment, said antibody has been changed to increase or decrease the avidity and/or affinity thereof.

In still another embodiment, said antibody has been changed to increase the production yield thereof.

In a further embodiement, said antibody has been changed to influence the pharmacokinetic properties thereof.

In still a further embodiment, said antibody has been changed to give new pharmacokinetic properties thereto.

In a further embodiment, said antibody is labeled and the binding thereof is inhibitable by an unlabeled form of said antibody and not by other binding

structures, and not inhibiting th binding of oth r binding structures having other specificities.

In another aspect the invention relates to a target structure displayed in, or on the surface of, tumour cells, said target structure

a) having the ability of being specifically blocked by and to specifically block the binding structure of an antibody as defined in any one of claims 1-14, and other binding structures with similar binding specificities,

b) being displayed in, and on the surface of, human gastrointestinal epithelial cells,

c) being displayed on a subpopulation of normal human gastrointestinal epithelial cells,

d) being highly expressed on the surface of tumour cells, and

e) being a target for cytotoxic effector mechanisms.

In one embodiment of said target structure, the binding structure is labeled and the binding thereof is inhibitable by an unlabeled form of said binding structure and not by other binding structures, and not inhibiting the binding of other binding structures having other binding specificies.

In a further embodiment of said target structure said binding structure comprises one or more of the complementarity determining region (CDR) sequences comprising essentially the amino acids number 23-33, 49-55, 88-98, 158-162, 177-193, 226-238 of the amino acid sequence shown in SEQ ID NO: 1, or other binding structures with similar unique binding properties.

In still a further embodiment of said target structure said binding structure is an antibody, which antibody in a further embodiment comprises the variable region of a light chain comprising essentially the amino acids number 1-109 of the amino acid sequence shown in SEQ ID NO: 1, and the variable region of a heavy chain comprising essentially the amino acids number 128-249 of the amino acid sequence shown in SEQ ID NO: 1.

Said target structure is in a furth r embodiment expressed homogenously in human colonic epithelial cells and less in pancreatic duct and bile duct cells.

In still a further embodiment, the expression of said target structure is correlated to gastrointestinal epithelial differentiation.

The invention relates in a further aspect to an anti-idiotype of a target structure as defined above, which anti-idiotype is specifically blocked by and specifically blocks a binding structure having similar binding specificity for said target structure.

In still a further aspect, the invention relates to a vaccine composition comprising as an active principle a target structure as defined above, or an anti-idiotype of said target structure as defined above.

In another aspect, the invention relates to a binding structure which recognizes a target structure as defined above, and which is of an organic chemical nature.

The invention is also related to a pharmaceutical composition comprising as an active principle an antibody as defined above.

In a further aspect the invention is related to a method of in vitro histopathological diagnosis and prognosis of human malignant disease, whereby a sample is contacted with an antibody as defined above and an indicator.

Embodiments of said method comprise tumour typing, tumour screening, tumour diagnosis and prognosis, and monitoring premalignant conditions

In still a further aspect, the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antigen comprising a target structure, as defined above, or an anti-idiotype of said target structure, as defined above, is assayed.

A further aspect of the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antibody as defined above is assayed.

A still further aspect of the invention is related to a method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of a complex of a) an antigen comprising a target structure, as defined above, or an anti-idiotype of said target structure, as defined above, and b) an antibody, as defined above, is assayed.

In a still further aspect, the invention is related to a method for in vivo diagnosis and prognosis of human malignant disease, whereby the localisation of an antibody, as defined above, to tumour deposits in a human subject is determined. Said antibody is preferably administered to the subject before the determination. In one embodiment said antibody is accumulated in tumour deposits. In a further embodiment, said method is quantitative.

Another aspect of the invention is related to a method for therapy of human malignant disease, whereby an antibody, as defined above, is administered to a human subject. In one embodiment of this method said antibody has been changed by being genetically linked to molecules giving the combined molecule changed pharmacokinetic properties. In another embodiment said antibody has been changed by being derivatised.

DETAILED DESCRIPTION OF THE INVENTION

The identification of novel tumour associated antigens (TAAs) is pivotal for the progression in the fields of tumour immunotherapy and diagnosis. In relation to the present invention, there was first developed, based on flow cytometric evaluation and use of a mini-library composed of specific antibody clones linked to different antibiotic resistance markers, methods for positive and subtractive selection of phage antibodies

employing intact cells as the antigen source. An scFv phage library (2.7×10^7) was constructed from a primate (*Macaca fascicularis*) immunised with pooled human colon carcinomas. This library was selected for three rounds by binding to Colo205 colon adenocarcinoma cells, and proteolytic elution followed by phage amplification.

Several antibodies reactive with colon carcinomas and with restricted reactivity with a few epithelial normal tissues were identified by immunohistochemistry. One clone, A3 scFv, recognised an epitope that was homogeneously expressed in 11/11 of colon and 4/4 pancreatic carcinomas studied and normal tissue expression restricted to subtypes of epithelia in the gastrointestinal tract. The A3 scFv had an apparent overall affinity about 100-fold higher than an A3 Fab, indicating binding of scFv homodimers. The cell surface density of the A3 epitope, calculated on the basis of Fab binding, was exceptionally high, approaching 3 million per cell.

Efficient T cell mediated killing of colon cancer cells coated with A3 scFv fused to the low MHC class II binding superantigen mutant SEA(D227A) is also demonstrated. The identified A3 molecule thus represents a TAA with properties that suggests its use for immunotherapy of colon and pancreatic cancer.

DISCUSSION

In relation to the present invention, efficient protocols for phage selection to be used for the identification of cell phenotype specific antibody fragments from large phage libraries was developed. The target specificities for the applications as exemplified were for colon tumour associated antigens.

First the frequency of pIII-scFv fusion protein surface display in the phage population using the herein presented phagemid construct for phage propagation was analysed. A higher level of C215 scFv display was achieved as compared to previous reports. This should

favour subtractive selection efficiency, but also increases the probability of avidity selection of low affinity antibodies from libraries.

Specificity of C215 scFv phage binding to colon adenocarcinoma Colo205 cells was clearly demonstrated. Bound phage could be efficiently eluted by use of the protease Genenase that specifically cleaves a target sequence between the phage protein III and the scFv antibody leaving the cells intact after elution. This non-chemical elution method should equally efficiently elute phage antibodies irrespectively of their binding affinity and only phage bound by scFv interactions, adding to the specificity of the process.

The enrichment achieved after three selection rounds on Colo205 cells (500 000×) using this selection protocol was similar to that reported by other investigators for selections on complex antigens.

After verifying the performance of the various methodological steps the combined technology was applied to library selections using Colo205 cells.

The library was constructed from a near human species immunised with human tumours. The antibody pool generated this way would potentially include affinity matured antibodies to tumour specific antigens in a limited background of xeno reactivities to widespread normal human tissue antigens (6). The antibodies identified recognised tumour and tissue differentiation antigens with restricted normal tissue distribution. All of the selected antibodies identified as colon cancer tissue reactive in the primary screening also reacted with viable Colo205 cells in flow cytometry. This restriction to cell surface specificities should reflect the selection process and not the composition of the library, since a suspension of a mixture of tumour tissue components was used for the immunisation.

In a similar previous study extra- and intracellular specificities were identified in an anti-melanoma library

produced the same way and s l cted using tissue sections as the antigen source (4). Tissue sections of resected human colorectal tumours and normal colon (mounted in the same well) were used for the primary screening using immunohistochemistry to assure the clinical relevance of the selected specificities, to increase the efficiency and to obtain more qualitative information as compared to flow cytometric screening.

The selected antibodies could be classified into four antibody specificity groups, distinguished by their reactivity patterns to epithelia in different organs (see Example 1, Table 1). Among these specificity groups, A3 scFv identified the most tumour selective antigen. This A3 TAA was highly, homogeneously and frequently expressed in samples of primary and metastatic colon cancer and of pancreatic cancer. Furthermore, its cell surface expression level as determined with the A3 Fab fusion protein (3 millions epitopes/cell) was exceptionally high and permissive for cell surface mediated cytotoxic effects.

Few, if any, of the frequently expressed human tumour antigens defined are tumour specific, but are commonly related to tissue differentiation such as A3 and the Ep-CAM. However, upregulated expression of these antigens in tumours should provide a basis for a therapeutically active dose window. The availability from the circulation of normal tissue compartments expressing the antigen may also be more restricted due to limited capillary permeability and their site of expression in the body (e.g. the exposure of the apical side of gut epithelial cells to circulating antibodies should be very limited).

The clinical experience with the pan-epithelial Ep-CAM reactive 17-1A mAb supports the feasibility to identify an effective non-toxic antibody dose. The restricted expression in epithelia of all of the selected scFv clones in this work, indicate that these clones in

princip l c uld be evaluated as candidates for immunotherapeutic applications analogously to the 17-1A, .g. as full-length mAbs. However, a particular advantage for the A3 TAA as compared to the Ep-CAM is the lack of expression in most normal epithelia such as of the lung and kidney, although the expression in the colon is similar.

The tissue distribution to subtypes of normal epithelia is supported by the selective expression in subtypes of carcinomas originating from the gastro-intestinal tract (see Example 2, Table 2).

Several of the previously well-known colon cancer associated antigens (CEA, CA50, CA19-9, CA242, Tag-72)(3) are expressed equally or more restrictedly in normal tissues as compared to the A3 epitope. However, in contrast to the A3 and the C215 Ep-CAM they are more heterogeneously expressed in tumours.

Use of antibodies to the Ep-CAM has demonstrated good clinical results including a survival advantage for colorectal cancer patients in an adjuvant setting (2). With the objective to induce tumour responses even in more advanced stage patients, the introduction of potent effector molecules in conjunction with this antibody will challenge the "normal tissue resistance" seen in the treatment with the naked 17-1A mAb. Preclinically, this could be studied in model systems using toxin-conjugated antibodies specific to the murine version of this antigen or animals transgenic for human colon cancer associated antigens.

Previously, antibody immunotoxins have been successfully used to cure mice in models with metastatically growing tumours expressing xeno (human) tumour antigens not expressed in mouse tissues (10). However, the TAAs used are truly tumour specific and the models do not reflect the potential for normal tissue targeted toxicity.

In previous studies we have reported the potential of superantigens as immunostimulatory toxins for tumour immunotherapy (8). Antibody mediated targeting of superantigens attracted large numbers of cytotoxic and cytokine-producing T cells to the tumour site. The superantigen SEA(D227A), mutated for low MHC class II binding affinity, was genetically linked to tumour targeting antibodies. This "tumour-selective" agent was applied to recruit T cells independent of MHC expression in the tumour, thus short-cutting the problems of MHC down regulation and polymorphism that represent significant obstacles for other active immunotherapeutic approaches.

The mini-library of the established "tumour-selective", 1F scFv phage, the "broadly-reactive" C215 phage and the non-specific D1.3 phage antibody clones was an essential tool for the development of protocols for efficient subtractive cell selection. A requirement for this selection principle is that the negative selection is followed by positive selection before phage rescue and amplification, due to the high frequency of non-displaying phage particles. Alternatively, non-displaying phage can be made non-infective by selective proteolysis (G. Winter, pers. comm.). Such a technique may allow the generation of "inert libraries", i.e. libraries that have been extensively negatively preselected (e.g. towards a cell in a resting state or a transfectable parental cell).

In conclusion, the "non-wanted" model phage specificity could selectively be subtracted from the phage population by a factor of approx. 100 for each selection round. Future subtractive selections using the developed protocol in combination with the use of large non-immune phage libraries for identification of differentially expressed cell surface antigens will demonstrate whether such an approach prove to be superior to the strategy we used in this study, i.e. positive

selection using an in vivo pre-selected immune repertoir , including restrictions and biases such as immunodominance (4). The low affinity and high epitope density demonstrated for the A3 Fab binding to tumour cells as compared to the A3 scFv fusion protein suggests formation of scFv multimers that interact with epitopes that cluster on cell surfaces. Higher affinity monovalent variants of A3 Fab or alternatively, stable divalent constructs such as full-length A3 Fv grafted mAbs compatible with the putative low immunogenicity of A3 should be developed. Such constructs would be suitable for targeting of appropriate effector molecules to this highly expressed gastro-intestinal tumour associated antigen.

The invention is further illustrated in the following nonlimiting experimental part of the description.

<u>EXMPERIMENTAL PART</u>

Materials and Methods

Animals

Cynomolgus Macaque (*Macaca fascicularis*) monkeys were kept and immunised at the Swedish Institute for Infectious Decease Control (SIIDC), Stockholm. Water and food were always available *ad libitum*. Four monkeys were immunised subcutaneously with 2 ml of a crude suspension of colon cancer tissues in 10 % normal cynomolgus serum in PBS. Booster doses were given day 21, 35, and 49. Antibody responses were demonstrated in two monkeys where the antigen had been admixed with alum adjuvant. All animals were kept according to Swedish legislation and the experiments were approved by the local ethical committees.

Tissues and cells

Human tumours and normal tissue samples were obtained from Lund University Hospital and Malmö General Hospital, Sweden. The human colorectal cell line Colo205, the human B cell lymphoma cell line Raji and the murine B16 melanoma cell line were from the American Tissue

Culture Collection (ATCC, Rockville, MD). The mouse melanoma B16-C215⁺ cells transfected with the expression vector pKGE839 containing the Ep-CAM-1 gene (C215) has been described previously (9).

The human cells were cultured in RPMI 1640 medium (Gibco, Middlesex, UK) supplemented with 10% heat inactivated foetal bovine serum (Gibco) and 0.1 mg/ml gentamycin sulphate (Biological Industries, Kibbutz Beit Haemek, Israel). The mouse cells were cultured in medium additionally supplemented with 1 mM glutamine (Hyclone, Cramlington, UK), 5×10^{-5} M β-mercaptoethanol (ICN, Costa Mesa, CA), 0.2 % $NaHCO_3$ (Seromed Biochrome, Berlin, Germany), 1×10^{-2} M HEPES (HyClone, UT) and 1×10^{-3} M sodium pyrovate (HyClone). The cells were repeatedly tested for Mycoplasma contamination with Gene-Probe Mycoplasma T. C. test (San Diego, CA).

Phagemid vector and phage library construction

Total spleen RNA was extracted from one of the responding monkeys using an RNA isolation kit from Promega (Mannheim, Germany) and cDNA was amplified using an RNA PCR kit from PE Biosystems (Stockholm, Sweden). The primers for cDNA synthesis of lambda light chain and heavy chain genes and for the assembly of these genes to scFv genes have been reported previously (4). The scFv cDNA was ligated into a phagemid vector (4) in fusion with the residues 249-406 of the M13 gene III. The scFv-gIII gene was expressed from a *phoA* promoter and the resulting protein was directed by the *E. coli* heat stable toxin II signal peptide.

Repeated electroporations of 7 µg library vector with scFv gene inserts resulted in a total of 2.7×10^7 primary transformed *E. coli* TG-1 growing as colonies on minimal agar plates. The colonies were scraped from the plates and grown in 2×YT at 150 rpm and 37°C for 1h. The culture was superinfected with M13K07 helper phage (Promega) in 50 times excess. Ampicillin to a concentration of 100 mg/l was added and the culture grown for a

further hour. After addition of kanamycin to a concentrati n of 70 mg/l, the culture w s grown for 15 h at 30°C and 250 rpm. The phage particles were harvested from the culture supernatant using two repeated PEG/NaCl precipitations. The precipitated phage was resolved in PBS 1% BSA.

Western blot analysis

A two-fold dilution series of scFv-C215 phage particles (from an undiluted stock of PEG-precipitated/concentrated phage) was applied to separation on a reducing 12% polyacrylamide gel with 1% SDS and 2% β-mercaptoethanol. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by electrophoresis. The membrane was blocked with 5% low-fat milk (Semper AB, Stockholm, Sweden) and then incubated with a rabbit antiserum against a protein III derived peptide sequence, AEGDDPAKAAFNSLQASATEC, conjugated to keyhole limpet hemocyanin. Secondary horse radish peroxidase (HRP) conjugated goat-anti-rabbit antibodies (Bio-Rad) were incubated for 30 min. Between all steps the membrane was washed 3 times during 5 min in PBS/ 0.5% Tween 20. The membrane was incubated in substrate (Amersham Pharmacia Biotech, Little Chalfon Buckinghamshire, UK) for one min. A light sensitive film (ECL hyperfilm, Amersham) was exposed to the membrane and developed for 0.5-5 min.

Similarly, to analyse the integrity of purified Fab (A3, including cynomolgus CH1 and Clambda domains), scFv- and Fab (including murine CH1 and Ckappa)-SEA(D227A) fusion proteins (produced as described previously (9)), 12% SDS-PAGEs were performed. The membranes with transferred proteins were incubated with purified polyclonal rabbit anti-SEA antibodies followed by the reagent steps described above.

Model and library phage selection on cells

Phage suspensions of the lambda light chain library (or of model phage), 10^{12} in 100 µl PBS/1% BSA, were

incubated with 3 million Colo205 c lls for 1h on ice. The cells were washed 3 times including a 10-min incubation using 2 ml PBS/1% BSA for each wash. The phage were eluted by adding 50 µl of 33 µg/ml Genenase to the cell pellet and incubated for 15 min. Genenase, which is a subtilisin BPN' mutant, S24C/H64A/E156S/G169A/Y217L, was kindly provided by Dr. Poul Carter (San Francisco, CA). After centrifugation the supernatant was transferred to a new tube and 250 µl 1% BSA in PBS was added. To rescue and amplify the selected library (and the model phage particles in the multi-pass experiment), the eluted phage particles were allowed to infect 1 ml, *E. coli* DH5αF' ($OD_{600\ nm}$ = 1.0). The infected bacterial culture was diluted 100 times with 2×YT supplemented with the proper antibiotic and cultured until an OD >1.0 (up to two days).

Finally, to produce soluble scFv the amber suppressor strain HB2151 of *E. coli* was infected with the selected library from the second and third round. After growth on agar plates containing ampicillin, single colonies were cultured in 96 Micro well plates in 2×YT medium supplemented with ampicillin at 30°C for 17 h. After centrifugation, removal of the supernatant to which an equal volume of PBS/1%BSA was added, individual scFvs were analysed for immunoreactivity to sections of human tumours and normal tissues. Briefly, the C-terminal tag, ATPAKSE, was detected using a rabbit antiserum followed by biotinylated goat anti-rabbit antibodies (DAKO A/S, Copenhagen, Denmark) and StreptABComplex HRP (DAKO A/S) (see "Immunohistochemistry").

Immunohistochemistry

Frozen cryosections (8 µm) were air-dried on slides, fixed in acetone at -20°C for 10 min and rehydrated in 20% foetal bovine serum in PBS (FBS). Endogenous biotin was blocked with avidin (diluted 1/6) for 15 min and then with biotin (diluted 1/6) for 15 min (Vector Laboratories, Burlingame, CA). Affinity purified and

biotinylated rabbit anti-SEA antibodi s, 5 µg/ml, were incubated for 30 min followed by StreptABComplex HRP (DAKO A/S, Copenhagen, Denmark), 1/110 diluted in 50 mM Tris pH 7.6 for 30 min. Between all steps the sections were washed 3 times in TBS. The staining reaction was developed for 8 min in 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma) dissolved in Tris pH 7.6 with 0.01 percent H_2O_2. After 10 min counterstaining in 0.5% methyl green, the slides were rinsed for 10 min in tap water and gradually dehydrated in 70-99% ethanol and xylene before mounting in DPX medium (Sigma).

Flow cytometry

The Colo205 colon cancer cells were dissociated with 0.02% w/v EDTA and washed with PBS. To follow the development of an antibody response in the monkeys the cells were incubated consecutively with diluted serum, for 1h at 4°C, biotinylated rabbit anti-human IgG antibodies (Southern Biotechnology Ass. Inc., Al, USA) for 30 min, and finally with avidin-PE (Becton Dickinson, Mountain View, CA) for 30 min.

The binding of model phage to the cells was analysed using rabbit-anti-M13 antibodies (produced by immunisation of rabbits with M13 particles) and FITC conjugated donkey anti-rabbit antibodies (Amersham Pharmacia Biotech). The binding of antibodies fused to SEA(D227A) was detected using biotinylated rabbit anti-SEA antibodies and avidin-PE. All reagents were diluted in PBS/1% BSA. The cells were washed twice with PBS/1% BSA after incubations with reagents and three times including 10 min incubations after binding of phage particles.

Flow cytometric analysis was performed using a FACSort flow cytometer (Becton Dickinson).

Affinity determination on cultured cells

A3 scFv-SEA(D227A), A3 Fab-SEA(D227A) and 1F scFv SEA(D227A) fusion proteins, 80 µg of each protein, were labelled with iodine as described by Bolton and Hunter to a specific activity of 10-15 µCi/µg. Colo205 cells and Raji cells, 30 000/sample were incubated with the iodinated fusion protein at 100 µl/tube in a two-fold dilution series in 1% BSA for 1h and then washed three times in PBS before measuring bound activity. The concentration of added and bound fusion protein was used for Scatchard analysis. The background binding to the Raji cells was subtracted to calculate the specific binding to the Colo205 cells.

Cytotoxicity assay

The T cell dependent cytotoxicity of the superantigen fusion protein (superantigen antibody dependent cellular cytotoxicity, SADCC) was measured in a standard 4 h chromium-release assay employing ^{51}Cr-labelled Colo205 cells as target cells and human T cells as effector cells (9). The percent specific lysis was calculated as:

$$100 \times \frac{\text{cpm experimental release} - \text{cpm background release}}{\text{cpm total release} - \text{cpm background release}}$$

EXAMPLE 1

Generation of tumour binding monoclonal cynomolgus antibodies

Cynomolgus monkeys, *Macaca fascicularis* (four individuals) were repeatedly immunised with a suspension of human colon carcinomas four times every other week. The gradual development of an antibody response in the monkeys was followed by flow cytometric staining of cultured colorectal cells, Colo205, using dilution series of the preimmune and immune sera. An IgG antibody response was elicited only when alum precipitated tumour tissue suspensions were used (two individuals).

Th monkey with the high st binding lev l of immune to preimmune serum antibodies was used for the construction of a large combinatorial scFv phage library of approximately 2.7×10^{7} (estimated from the number of primary transformants). The primate phage library was selected using Colo205 cells. The total phage yield (eluted/added number of phage counted as colony forming units, CFU) from three consecutive selection rounds increased gradually from 1.9×10^{-7}, 1.4×10^{-5}, to 1.2×10^{-3}. Five percent (12/246) of the monoclonal soluble scFv:s produced from the phage library after the third round of selection were demonstrated to bind to sections of a human colorectal cancer tissue and to intact Colo205 cells by flow cytometry. All of the selected antibodies demonstrated individually unique nucleic acid sequences according to Hinf I restriction patterns analysed by 1% agarose gel electrophoresis.

The antibody genes were amplified by polymerase chain reaction using 5 µl of bacterial cultures and primers complementary to regions 5′and 3′ to the scFv gene in the phagemid vector (regions in the *pho*A promoter and in the M13 gene III).

The selected scFv demonstrate individually unique reactivity with epithelia in normal tissues

The colorectal cancer reactive scFv's were classified into specificity groups based on their immunohistochemical reactivity pattern with normal tissues (Table 1). The antibodies studied in detail were A3 scFv (and A3 scFv-SEA(D227A)), A10 scFv, 3D scFv and 1D scFv. The representative antibodies could be distinguished from each other by their fine specificity to epithelia in different organs and by their binding to leukocytes. The 1D scFv strongly reacted with gut epithelia and was the only antibody that reacted with cells of polymorph nuclear granulocyte morphology. The 1D scFv also differed from the other antibodies by staining the luminal surface of kidney tubuli and collecting ducts

whereas the A10 scFv reacted homogeneously (non-polarly) with these epithelial cells and 3D scFv and A3 scFv were negative. 1D, A10 and 3D, but not A3 scFv also reacted with macrophage-like cells in the lung.

A fifth group of antibodies, not extensively evaluated and thus not included in Table 1, reacted with colon epithelia, leukocytes and Kuppfer cells in the liver. The A3 scFv stands out as demonstrating the most restricted reactivity with the panel of normal tissues used. The most prominent normal tissue reactivity of the A3 was staining of normal colon epithelium. Weak staining were also detected in small ducts of the pancreas and bile ducts of the liver and of substructures in small bowel epithelia. The surface epithelium of one of the two stomach samples was strongly stained by the A3 antibody.

The reactivity pattern of the A3 scFv was confirmed using the fusion protein A3 scFv-SEA(D227A). This format permitted the use of polyclonal rabbit anti-SEA antibodies for immunohistochemical detection, which is a more sensitive detection system demonstrating lower background and tissue crossreactivity as compared to the use of secondary antibodies to the peptide tag, ATPAKSE, at the C-terminus of the scFvs.

Table 1 Immunohistochemical reactivity to normal human tissues of soluble scFv fragments from the selected colorectal cancer phage library

		scFv clone designation			
Tissue / sub-structure	n*	A3 **	A10	3D	1D
Esophagus / epithelial tissue	1	0	ND	ND	ND
/ non-epithelial tissue		0	ND	ND	ND
Colon / epithelium	5	++	+	+	++
/ non-epithelial tissue		0	0	0	granulocytes ++
Small bowel / villi epithelium	2	(+)	heterogenously +	+	heterogenously (+)
/ basal glandulae		+	+	+	++
/ non-epithelial tissue		0	0	0	0
Ventricle / surface epithelium	2	0, ++	0	0, +	++
/ glandular epithelium		0	+, ++	0	++
/ non-epithelial tissue		0	0	0	0
Pancreas / acini	1	0	(+)	+	++
/ small ducts		(+)	(+)	+	++
/ large ducts		0	(+)	+	++
/ non-epithelial tissue		0	0	0	0
/ endocrine		0	0	0	0
Liver / hepatocytes	2	0	ND	ND	ND
/ Kuppfer cells		0	ND	ND	ND
/ bile ducts		(+)	ND	ND	ND
Kidney / proximal tubuli	1	0	+	0	luminal surface ++
/ distal tubuli		0	+	0	luminal surface ++
/ collecting ducts		0	+	0	luminal surface ++
/ glomeruli		0	0	0	0
/ non-epithelial tissue		0	0	0	0
Bladder / epithelial tissue	1	0	ND	ND	ND
/ non-epithelial tissue		0	ND	ND	ND
Prostate / epithelial tissue	1	0	++	+	and secreted material ++
/ non-epithelial tissue		0	0	0	0
Lung / bronchial epithelium	1	0	(+)	(+)	0
/ alveolar epithelium		0	(+)	(+)	0
/ non-epithelial tissue		0	macrophages +	macrophages +	granulocytes ++, macrophages +
CNS / gray matter	1	0	ND	ND	ND
/ white matter		0	ND	ND	ND
Skeletal muscle	1	0	ND	ND	ND

Notes t Table 1
0 = negative, (+) = weak, + = moderate, ++ = strong, ND = not determined
* Number of tissue samples examined
** The reactivity of A3 scFv has been confirmed with the A3 scFv SEA(D227A) fusion protein

EXAMPLE 2

The A3 tumour-associated antigen is homogeneously and frequently expressed in colorectal and pancreatic tumours

The A3 scFv-SEA(D227A) fusion protein was used for immunohistochemical staining of various tumours of epithelial origin (Table 2 and Figure 1). The fusion protein homogeneously and strongly stained 11/11 samples of primary colon cancer tissues and 4/4 metastatic colon cancer samples resected from the ovary, a lymph node and the liver. Pancreatic cancer tumours, 4/4 samples, were equally strongly positive. In contrast, tissue samples of gastric, prostate, breast and non-small cell lung carcinomas were negative.

Table 2 Tumor tissue reactivity of A3 scFv SEA(D227A)

Tumor tissue	n	Reactivity
Colon cancer, primary tumors	11	All tumor cells are strongly and homogenously stained
Colon cancer metastasis in lymph node, liver and ovary	4	As above
Pancreas cancer	4	As above
Ventricle cancer	2	Negative
Prostate cancer	2	Negative
Breast cancer	2	Negative
Lung carcinoma (non-small cell)	2	Negative
Malignant melanoma	2	Negative

EXAMPLE 3

The A3 TAA is highly expressed on the surface of colon cancer cells

The results from several Scatchard plots for affinity determination, based on the binding of the fusion proteins A3 scFv-SEA(D227A), A3 Fab and 1F scFv-SEA(D227A) (1F was classified to the A3 specificity group) to Colo205 cells, are summarised in Table 3. Specific binding was calculated by subtraction of non-specific binding to human B cell lymphoma Raji cells, a cell line not expressing the A3 and 1F TAAs, from the binding to Colo205 cells. Linear regression was used to calculate the slope and the intercept of the extrapolated line in the Scatchard plot. The A3 scFv-SEA(D227A) fusion protein saturated approximately 10-fold less binding sites per cell as compared to the A3 Fab (approx. 3 million sites per cell) fusion protein, indicating that divalent (multivalent) binding was involved for the scFv. This is supported by the more than 100-fold higher overall affinity (3.6-5.5 nM) for the A3 scFv fusion protein as compared to the A3 Fab (580-780 nM).

A single experiment performed using the 1F scFv-SEA(D227A) fusion protein, demonstrated similar binding affinity and saturation of binding sites as the A3 scFv-SEA(D227A) fusion protein.

Table 3 Scatchard analysis of iodinated fusion proteins binding to Colo205 cells

Fusion protein	n*	Kd (nM)	million sites /cell
A3 Fab-SEA(D227A)	2	580-780	3.0-3.9
A3 scFv-SEA(D227A)	3	3.6-5.5	0.11-0.39
1F scFv-SEA(D227A)	1	4.2	0.18

* Number of experiments performed

EXAMPLE 4

A3 and 1F scFv-SEA(D227A) mediate T cell lysis of Colo205 cells

The capacity of the two fusion proteins A3 and 1F scFv-SEA(D227A) to mediate superantigen antibody dependent cellular cytotoxicity (SADCC) towards Colo205 cells was investigated and compared with the positive control C215 Fab-SEA(D227A) and negative control D1.3 scFv-SEA(D227A) fusion proteins. The A3 scFv-SEA(D227A) fusion protein titration approached a plateau for maximal lysis which was similar, approx. 50 percent in this 4 h assay, to that demonstrated for the C215 Fab-SEA(D227A) fusion protein, although at a ten-fold higher concentration (Figure 2). The 1F scFv-SEA(D227A) mediated a similar level of cytotoxicity at a slightly higher concentration as compared to the A3 scFv-SEA(D227A).

The negative control D1.3 scFv SEA(D227A) fusion protein did not mediate any cytotoxicity.

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LEGENDS TO FIGURES

Figure 1 *The A3 tumour-associated antigen is homogeneously expressed in primary and metastatic tumours*

Immunohistochemical staining of frozen and acetone fixed sections of human tumour tissues using A3 scFv-SEA(D227A) and C215 Fab-SEA(D227A) at 70 nM. The A3 scFv fusion protein reacted strongly and homogeneously with both primary colon and pancreatic carcinoma resected from tumour patients. A representative staining of a primary colon cancer is shown for C215 Fab-SEA(D227A) in (A) and for A3 scFv-SEA(D227A) in (B). Staining by A3 scFv-SEA(D227A) of a colon cancer liver metastasis is shown in (C) and of a primary pancreatic cancer in (D).

Figure 2 *A3 scFv-SEA(D227A) coated Colo205 tumour cells are efficiently killed by T cells.*

Superantigen antibody dependent cellular cytotoxicity (SADCC) towards Colo205 cells mediated by A3 scFv-SEA(D227A) reached the same maximal cytotoxicity as the anti-Ep-CAM fusion protein C215 Fab-SEA(D227A), although at a ten-fold higher concentration. The absence of cytotoxicity mediated by the D1.3 scFv-SEA(D227A) demonstrates the need of a tumour targeting antibody moiety in the fusion protein.

CLAIMS

1. An antibody, or fragments thereof, having a binding structure for a target structure displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells, said binding structure comprising the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:1, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence shown in SEQ ID NO: 1, or other binding structures with similar unique binding properties.

2. An antibody according to claim 1, which is phage selected.

3. An antibody according to claim 1, wherein the sequences are of Macaca fascicularis origin.

4. An antibody according to claim 1, wherein the sequences have an identity of at least 84% to corresponding sequences of human origin.

5. An antibody according to claim 1, which has low immunogenicity or non-immunogenicity in humans.

6. An antibody according to claim 1, which has been derivatised by genetically linking to other polypeptides, and/or by chemical conjugation to organic or non-organic chemical molecules, and/or by di-, oligo- or multimerisation.

7. An antibody according to claim 1, which is genetically linked or chemically conjugated to cytotoxic polypeptides or to cytotoxic organic or non-organic chemical molecules.

8. An antibody according to claim 1, which is genetically linked or chemically conjugated to biologically active molecules.

9. An antibody according to claim 1, which is genetically linked or chemically conjugated to immune activating molecules.

10. An antibody according to claim 1, which has been changed to increase or decrease the avidity and/or affinity thereof.

11. An antibody according to claim 1, which has been changed to increase the production yield thereof.

12. An antibody according to claim 1, which has been changed to influence the pharmacokinetic properties thereof.

13. An antibody according to claim 1, which has been changed to give new pharmacokinetic properties thereto.

14. An antibody according to claim 1, which is labeled and the binding thereof is inhibitable by an unlabeled form of said antibody and not by other binding structures, and not inhibiting the binding of other binding structures having other binding specificities.

15. A target structure displayed in, or on the surface of, tumour cells, said target structure

a) having the ability of being specifically blocked by and to specifically block the binding structure of an antibody as defined in any one of claims 1-14, and other binding structures with similar binding properties,

b) being displayed in, and on the surface of, human gastrointestinal epithelial cells,

c) being displayed on a subpopulation of normal human gastrointestinal epithelial cells,

d) being highly expressed on the surface of tumour cells, and

e) being a target for cytotoxic effector mechanisms.

16. A target structure according to claim 15, wherein the binding structure is labeled and the binding thereof is inhibitable by an unlabeled form of said

binding structure and not by other binding structur s, and not inhibiting th binding of oth r binding structures having other binding specificities.

17. A target structure according to claim 15, wherein said binding structure comprises one or more of the complementarity determining region (CDR) sequences comprising essentially the amino acids number 23-33, 49-55, 88-98, 158-162, 177-193, 226-238 of the amino acid sequence shown in SEQ ID NO: 1, or other binding structures with similar unique binding properties.

18. A target sturcture according to claim 15, wherein said binding structure is an antibody.

19. A target structure according to claim 18, wherein said antibody comprises the variable region of a light chain comprising essentially the amino acids number 1-109 of the amino acid sequence shown in SEQ ID NO: 1, and the variable region of a heavy chain comprising essentiallay the amino acids number 128-249 of the amino acid sequence shown in SEQ ID NO: 1.

20. A target structure according to any one of claims 15-19, which is expressed homogenously in human colonic epithelial cells and less in pancreatic duct and bile duct cells.

21. A target structure according to any one of claims 15-20, the expression of which is correlated to gastrointestinal epithelial differentiation.

22. An anti-idiotype of a target structure as defined in any one of claims 15-21, which anti-idiotype is specifically blocked by and specifically blocks a binding structure having similar binding specificity for said target structure.

23. A vaccine composition comprising as an active principle a target structure as defined in any one of claims 15-21, or an anti-idiotype of said target structure as defined in claim 22.

24. A binding structure which recognizes a target structure as defined in any one of claims 15-21, and which is of an organic chemical nature.

25. A pharmaceutical composition comprising as an active principle an antibody as defined in any one of claims 1-14.

26. A method of in vitro histopathological diagnosis and prognosis of human malignant desease, whereby a sample is contacted with an antibody as defined in any one of claims 1-14 and an indicator.

27. A method according to claim 26, which method comprises tumour typing.

28. A method according to claim 26, which method comprises tumour screening.

29. A method according to claim 26, which method comprises tumour diagnosis and prognosis.

30. A method according to claim 26, which method comprises monitoring premalignant conditions.

31. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antigen comprising a target structure, as defined in any one of claims 15-21, or an anti-idiotype of said target structure, as defined in claim 22, is assayed.

32. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of an antibody as defined in any one of claims 1-14 is assayed.

33. A method for in vitro diagnosis and prognosis of human malignant disease, whereby concentrations in bodily fluids of a complex of a) an antigen comprising a target structure, as defined in any one of claims 15-21, or an anti-idiotype of said target structure, as defined in claim 22, and b) an antibody, as defined in any one of claims 1-14, is assayed.

34. A method for in vivo diagnosis and prognosis of human malignant disease, whereby the localisation of an

antibody, as defined in any one of claims 1-14, to tumour deposits in a human subject is det rmined.

35. A method according to claim 34, whereby said antibody is administered to the subject before the determination.

36. A method according to claim 34, whereby said antibody is accumulated in tumour deposits.

37. A method according to any one of claims 34-36, which is quantitative.

38. A method for therapy of human malignant disease, whereby an antibody, as defined in any one of claims 1-14, is administered to a human subject.

39. A method according to claim 38, whereby said antibody has been changed by being genetically linked to molecules giving the combined molecule changed pharmacokinetic properties.

40. A method according to claim 38, whereby said antibody has been changed by being derivatised.

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Fig 1.

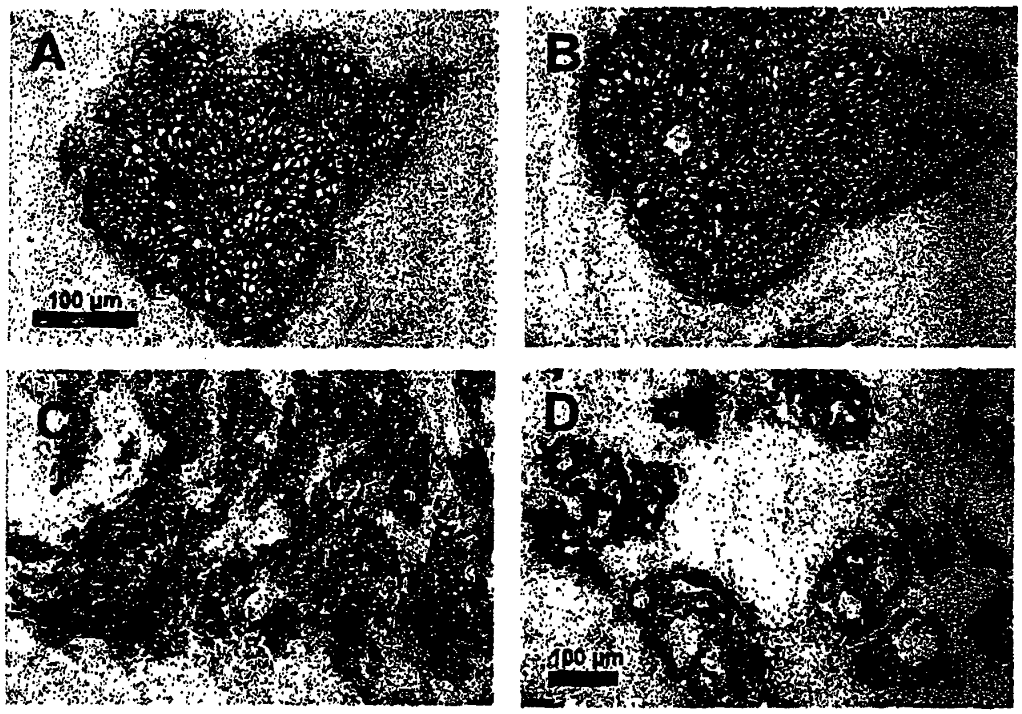
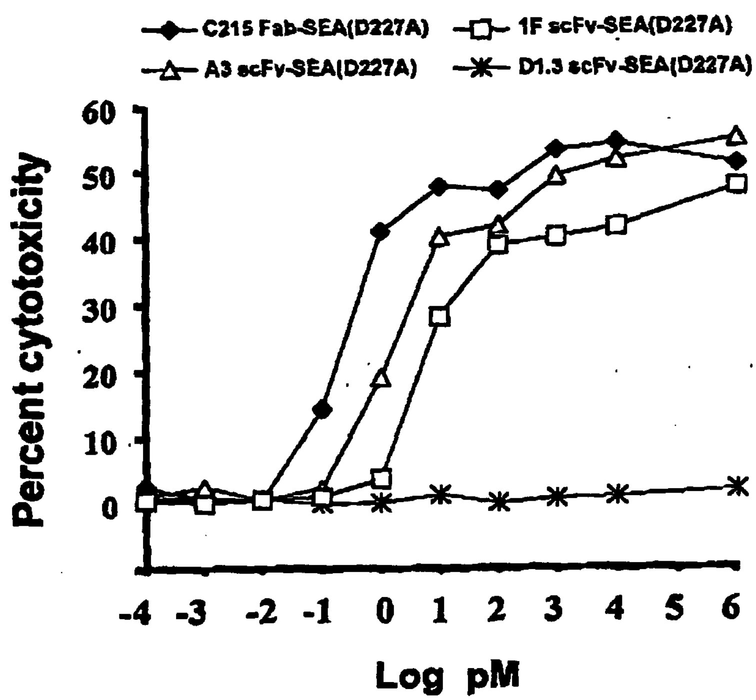


Fig. 2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1F scFv-SEA(D227A)
A3 scFv-SEA(D227A)
D1.3 scFv-SEA(D227A)
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30
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ABSTRACT

An antibody, or a fragment thereof, having a binding structure for a target structure is described. The antibody is displayed in, and on the cell surface of, human gastrointestinal epithelial tumour cells and in a subpopulation of normal human gastrointestinal epithelial cells. Said binding structure comprises the complementarity determining region (CDR) sequences in the light chain comprising essentially the amino acids number 23-33 (CDR1), 49-55 (CDR2), 88-98 (CDR3) of the amino acid sequence shown in SEQ ID NO:1, and the CDR sequences in the heavy chain comprising essentially the amino acids number 158-162 (CDR1), 177-193 (CDR2), 226-238 (CDR3) of the amino acid sequence shown in SEQ ID NO: 1, or other binding structures with similar unique binding properties.

There is also described a target structure displayed in, or on the surface of tumour cells, a vaccine composition comprising said target structure, a pharmaceutical composition comprising said antibody as well as methods related to human malignant diseases.

Elected for publication: Figure 1